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Inhibition of β-estradiol on trachea smooth muscle contraction *in vitro* and *in vivo*

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KEY WORDS estradiol; trachea; smooth muscle; muscle relaxation; muscle contraction; acetylcholine; histamine

ABSTRACT

AIM: To investigate the effect of β-estradiol on trachea smooth muscle contraction *in vitro* and *in vivo*. **METHODS**: (1) Rabbit tracheas were incubated in organ baths filled with Krebs solution and supplied with a mixed gas of 95 % O_2 and 5 % CO_2. The isometric force was measured by ink-writing recorders. (2) The incubation period of asthma induced by histamine and acetylcholine (ACh) in guinea pig were measured before and after β-estradiol (1 mg/kg) were given intramuscularly. **RESULTS**: (1) Administration of β-estradiol (0.1 mmol/L) caused relaxation of isolated trachea muscle strips (TMS) in rabbits pre-contracted by ACh and KCl (39 % ± 5 % and 45 % ± 19 %). The presence of indomethacin or methylene blue partly decreased the relaxation to β-estradiol (26 % ± 8 % and 28 % ± 13 %), but N_ω-nitro-*L*-arginine (*L*-NNA) and propranolol and epithelium removal did not affect it (38 % ± 10 %, 40 % ± 15 %, 37 % ± 8 %). β-Estradiol can shifted the concentration-response curves of ACh and $CaCl_2$ to the rightward (pD_2' = 3.98 and 4.75). In addition, it could also significantly inhibit the contraction of phase Ⅰ caused by ACh, but did not affect the contraction of phase Ⅱ caused by $CaCl_2$. (2) The incubation period of asthma in guinea pig were delayed by β-estradiol (1 mg/kg) given intramuscularly. **CONCLUSION**: (1) The relaxation of β-estradiol *in vitro* was epithelium independent and associated with the inhibition of potential-dependent channel and release of Ca^{2+} from sarcoplasm reticulum induced by ACh. In addition, release of prostaglandins from trachea smooth muscle cells and relaxation through cGMP approach were also included. β-Adrenoceptor-mediated relaxation was not involved. (2) β-Estradiol can relax the trachea *in vivo* in guniea pig.

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INTRODUCTION

It is observed that asthma can be induced or can deteriorate during menopause. This phenomena may be associated with the decrease of estrogen level in menopausal women. Consequently, estrogen was applied to the treatment of the asthmatic patients with moderate or severe menopause syndrome[1] and its therapy effect was obviously. There are a lot of investigations into the effect of estrogen on vascular smooth muscle. Its mechanism includes the inhibition of potential-dependent channel (PDC) and decreased influx of extracellular Ca^{2+}[2], increasing NO, prostaglandins released from epithelia *et al*[3]. However, the mechanism of estrogen on airway smooth muscle has not been reported either abroad or at home. In order to study the mechanism of the therapy of estrogen, we investigated its effect on trachea *in vivo* and *in vitro*.

MATERIALS AND METHODS

Drugs and instruments The following drugs were used: β-estradiol, N_ω-nitro-*L*-arginine (*L*-NNA) (Sigma Chemical Co, USA); acetylcholine (ACh) and propranolol (Beijing 2th Pharmaceutical Co); indomethacin (Jiangsu Taicang Pharmaceutical Co); methylene blue (Chemical Industry of Antichemical College); histamine (Shanghai Lizhu Dongfeng Biology Technical Corporation). β-Estradiol was prepared by dissolving in 1, 2-propanediol or in sesame oil. Indomethacin was prepared by dissolving in 20 % ethanol. Krebs solution contains (in mmol/L): NaCl 120.6, KCl 5.9, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, $CaCl_2$ 2.5, $C_6H_{12}O_6$ 11.5. Composition of Krebs solution free of Ca^{2+} is the same as that of Krebs' except

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that $CaCl_2$ was replaced by editic acid 0.1 mmol/L.

Biomedical transducers were chosen from Institute of Space Medico-Engineering, and 2D ink-writing recorders were from the Chengdu Instrument Plant.

Animals and tissue preparation Male rabbits, weighing 2.5 kg ± 0.5 kg (supplied by Experimental Animal Center of Lanzhou Medical College) were stunned and exsanguinated. The trachea was dissected out and the connective tissues were gently removed. The trachea rings, 5 mm in width, were excised in a transverse orientation. Then the rings were cut longitudinally alone the ventral surface into strips. The epithelia of trachea were removed by rubbing gently the luminal surface with cotton swab. (At the end of the experiment, arbitrarily selected strips were fixed in 10 % formalin for histologic examination to confirm the presence of removal epithelia[4]). Each isolated trachea muscle strips (TMS) was suspended in organ baths containing 5 mL Krebs solution (37 ℃) and supplied with a mixed gas of 95 % O_2 and 5 % CO_2. The strips equilibrated under a resting tension of 2 g for at least 80 min with replacement of the bath fluid every 20 min. The isometric tension of TMS was recorded continuously through the force transducers.

Male guinea pigs, weighing 180 – 200 g (supplied by Experimental Animal Center of Lanzhou Medical College) were chosen. In the first day, sesame oil were given to each guinea pig intramuscularly. One hour later every two guinea pigs were placed into a chamber (its volume is 4.0 L) and the mixture of 2 % ACh and 0.1 % histamine were sprayed into it continuously under the pressure of 53 kPa for 20 s. The incubation period of asthma (until the guinea pigs fell down) were observed for 10 min and recorded as control group. In the following day, pigs falling down in 2 min were chosen and experiments were repeated after β-estradiol (1 mg/kg) were given intramuscularly for 1 h[5] and incubation period was recorded as β-estradiol group.

Inhibition of β-estradiol on contraction of TMS pre-contracted with ACh and KCl Following the equilibration period, the TMS (with or without epithelium) were pre-contracted with ACh (10 μmol/L). After the contraction evoked had reached a plateau, the strips were washed by Krebs solution (37 ℃). Thirty min later, the strips were pre-contered again. When a stable contraction was obtained, the β-estradiol (100 μmol/L) were administered. In another group, the experiment was repeated with KCl (80 mmol/L) in epithelium intact group.

Influence of antagonists on effect of β-estradiol In the epithelium intact group, after having been incubated with indomethacin (10 μmol/L), methylene blue (10 μmol/L), *L*-NNA (10 μmol/L) and propranolol (30 μmol/L) for 20 min, the strips were contracted by ACh (10 μmol/L). When the contraction reached the plateau, β-estradiol (100 μmol/L) was added.

Inhibition of β-estradiol on concentration-response curves of ACh and $CaCl_2$ In one group, after concentration-response curves were obtained by cumulative addition of ACh (0.1 μmol/L – 100 μmol/L), the strips were washed by Krebs and equilibrated for 30 min. Then, concentration-response curves were regenerated after the strips had been incubated with β-estradiol (10 μmol/L, 50 μmol/L, and 100 μmol/L) for 20 min. In another group, after the TMS strips had been equilibrated in Ca^{2+}-free Krebs solution for 80 min, KCl (80 mmol/L) was added for 5 min to depolarize the membrane of TMS cell and concentration-response curves of $CaCl_2$ (10 μmol/L – 10 mmol/L) were established. Then the strips were also washed and equilibrated. Following the incubation of β-estradiol (10 μmol/L) for 20 min, the concentration-response curves of $CaCl_2$ were repeated.

Inhibition of β-estradiol on two phases contraction induced by ACh and $CaCl_2$ After preparations had been incubated in Krebs solution free of Ca^{2+} for an hour, a quick, short contraction (phase Ⅰ) was obtained with administration of ACh 10 μmol/L. When the contraction reach a plateau, $CaCl_2$ 10 mmol/L was added immediately and another contraction (phase Ⅱ) appeared. Having been washed for 30 min, preparations were incubated with β-estradiol (10 μmol/L) and the same experiment was repeated 20 min later.

Statistical analysis The data were expressed as $x \pm s$ and analyzed by t test or ANOVA and P value < 0.05 was considered significant.

RESULTS

Inhibition of β-estradiol on isolated trachea *smooth muscle in rabbits in vitro*

Inhibition of β-estradiol on contraction of TMS pre-contracted with ACh and KCl Administration of β-estradiol (100 μmol/L) induced relaxation of TMS pre-contractions with ACh (epithelium-intact and epithelium-denuded group) and KCl, and relaxation percentages were respectively 39 % ± 5 %, 37 % ± 8 %, 45 % ±

19 %. There was no significant difference between epithelium-intact and epithelium-denuded group ($P > 0.05$). All the data were expressed as percent change in tension from contraction induced by ACh (10 μmol/L) and KCl (80 mmol/L) (Tab 1).

Tab 1. Effect of β-estradiol 100 μmol/L and antagonists on the contraction in isolated rabbits trachea induced by ACh. $^bP < 0.05$, $^cP < 0.01$ *vs* epithelium-intact group.

Group	*n*	Relaxation/%
β-Estradiol (epithelium-intact)	9	39 ± 5
β-Estradiol (epithelim-denuded)	6	37 ± 8
β-Estradiol + Methylene blue (10 μmol/L)	9	28 ± 13^b
β-Estradiol + Indomethacin (10 μmol/L)	9	26 ± 8^c
β-Estradiol + Propranolol (30 μmol/L)	9	40 ± 15
β-Estradiol + *L*-NNA (10 μmol/L)	9	38 ± 10

Influence of antagonists on effect of β-estradiol The relaxation responses to β-estradiol were partly decreased by the presence of methylene blue or indomethacin (compared with epithelium-intact group, $P < 0.05$ or $P < 0.01$), but were not affected by the presence of *L*-NNA and propranolol (Tab 1).

Inhibitions of β-estradiol on concentration-response curves of ACh and $CaCl_2$ Increasing concentration of ACh or $CaCl_2$ resulted in concentration-dependent contraction of TMS (pD_2 values were 5.27 and 2.95 respectively). However incubation of β-estradiol (10 μmol/L, 50 μmol/L or 100 μmol/L) for 20 min shifted the concentration-response curves of ACh and $CaCl_2$ to the right in a unparalleled manner with decreasing the maximal response (pD_2' values were 3.98 and 4.75 respectively). The rightward shift of the concentration-response curves of ACh were dose dependent. All data were expressed as a percentage of the maximal response to ACh and $CaCl_2$ (Fig 1).

Inhibitions of β-estradiol on two phases contraction In Ca^{2+}-free Krebs solution, ACh induced contraction of phase Ⅰ (0.94 g ± 0.25 g) which was caused by the release of intracellular Ca^{2+}, and subsequent administration of $CaCl_2$ induced contraction of phase Ⅱ (0.89 g ± 0.21 g) which was caused by influx of extracellular Ca^{2+} through the receptor-operated channels. Incubation of β-estradiol (100 μmol/L) for 20 min significantly inhibited the contraction of phase Ⅰ (0.36 g ± 0.13 g), but did not affect the contraction of phase Ⅱ (0.85 g ± 0.26 g) (Fig 2).

Inhibition of β-estradiol on incubation period of asthma induced by ACh and histamine in guinea pigs in vivo After giving β-estradiol intramuscularly, the incubation period of asthma induced by 2 % ACh and 0.1 % histamine were significantly prolonged ($P < 0.05$, Tab 2).

Tab 2. Inhibition of β-estradiol on incubation period of asthma induced by ACh and histamine in guinea pigs *in vivo*. $^cP < 0.01$ *vs* control.

Group	*n*	Incubation period/s
Control	6	90 ± 14
Estradiol (1 mg/kg)	7	145 ± 35^c

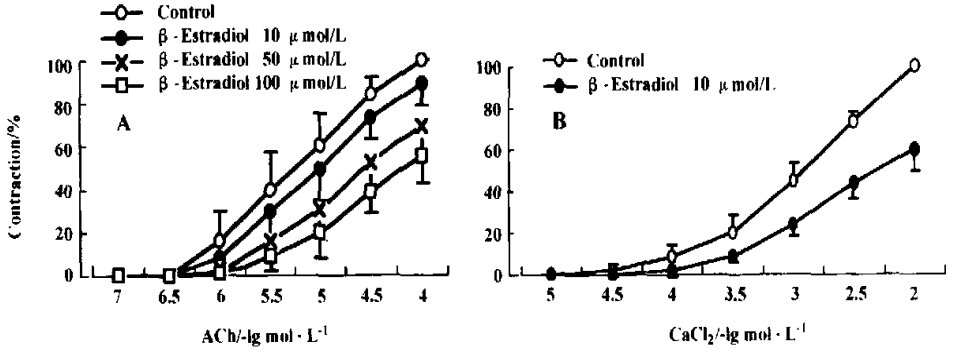

Fig 1. Inhibitions of β-estradiol on ACh (A) and $CaCl_2$ (B) concentration-response curves in isolated trachea smooth muscle in rabbits. $n = 9$. $\bar{x} \pm s$.

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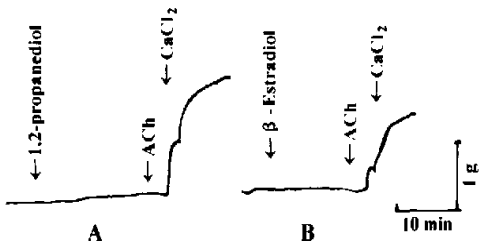

Fig 2. Inhibition of β-estradiol on two- phase contraction induced by ACh in isolated rabbit trachea. (A) control group; (B) β-estradiol group. $n=8$. $P<0.01$ *vs* control group. All the data were expressed as force tension(g).

DISCUSSION

In vitro experiments showed that β-estradiol obviously relaxed the contractioninduced by ACh. The relaxation was weakened by the presence of indomethacin (an inhibitor of cyclooxygenase), but was not affected by the presence of *L*-NNA (NO synthase inhibitor) nor by the epithelium removal. Since TMS tension can be modulated by epithelium derived relaxing factors (EpDRF) which may include NO and cyclooxygenase products such as PGE_2 and PGI_2. Meanwhile, NO and cyclooxygenase products can also be released from airway smooth muscle cells[6]. These results indicate that relaxation of β-estradiol was epithelium independent and did not contribute to the release of NO, but partly to the release of prostaglandins from trachea smooth muscle cells. After incubation with methylene blue (a potent inactivator of guanylate cyclase), the relaxation responses of β-estradiol significantly decreased. Whilst incubation with propranol, an adrenoreceptor antagonist, did not affect it. cGMP is an important second messenger mediating tracheal relaxation and β-adrenoceptor-mediated relaxation is another important approach of relaxation of TMS[7]. The results suggested that cGMP-related relaxant were partly involved in, but β-adrenoceptor-mediated relaxation was not.

Contraction of TMS induced by KCl was also relaxed by administration of β-estradiol. Furthermore, concentration-response curve of $CaCl_2$ was shifted to the rightward by incubation with β-estradiol. The two results demonstrated that β-estradiol substantially inhibits the extracellular Ca^{2+} influx through PDC.

Two-phase contraction induced by ACh involves intracellular Ca^{2+} and extracellular Ca^{2+} [8]. On the one hand, activation of M_3[9] receptor results in an increase of inositol triphosphate and release of intracellular Ca^{2+} from the sarcoplasmic reticulum. On the other hand, the receptor-operate channel can be opened and influx of extracellular Ca^{2+} is increased. In Ca^{2+}-free Krebs solution, release of Ca^{2+} from the sarcoplasmic reticulum is responsible for the contraction of phase Ⅰ induced by ACh, whereas influx of extracellular Ca^{2+} is for the contraction of phase Ⅱ with administration of $CaCl_2$[10]. Incubation with β-estradiol significantly inhibited the contraction of Phase Ⅰ. Inhibition of β-estradiol on the contraction induced by ACh, the concentration-response curve of ACh, and the contration of phase Ⅰ indicated that β-estradiol inhibited the release of intracellular Ca^{2+} and did not affect the contraction depending on influx of extracellular Ca^{2+} through receptor-operate channel.

The experiment *in vivo* showed that β-estradiol can prolong the incubation period of asthma induced by ACh and histamine in guinea pigs. It demonstrated that β-estradiol relaxed airway smooth muscle *in vivo*.

In conclusion, β-estradiol can relax trachea smooth muscle both *in vitro* and *in vivo*. The relaxation *in vitro* was epithelium-independent. The mechanism was associated with the inhibition of PDC opened by KCl and release of Ca^{2+} from sarcoplasm reticulum induced by ACh. In addition, release of prostaglandins by TMS cells and cGMP-related relaxants were also involved. However β-adrenoceptor-mediated relaxation was not included.

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雌二醇对离体和在体气管平滑肌收缩活动的抑制作用

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关键词 雌二醇；气管；平滑肌；肌松弛；肌收缩；乙酰胆碱；组胺

目的：研究雌二醇对离体和在体气管平滑肌收缩的作用. **方法**：(1) 将家兔离体气管平滑肌条置于装有 Krebs 液的肌槽中温育，并通入 95 % O_2 和 5 % CO_2 的混合气体. 二导记录仪记录肌条的等长张力. (2) 测量肌注雌二醇 (1 mg/kg) 前后乙酰胆碱和组胺引发豚鼠哮喘的潜伏期. 结果：(1) 雌二醇 (100 μmol/L) 对乙酰胆碱和氯化钾诱发的收缩有明显的舒张作用（舒张百分比分别为 39 % ± 5 % 和 45 % ± 19 %）. 其作用可被吲哚美辛和亚甲蓝部分阻断（26 % ± 8 % 和 28 % ± 13 %），但不能被 *L*-NNA、心得安和去除上皮所影响（舒张百分比分别为 38 % ± 10 %，40 % ± 15 %，37 % ± 8 %）. 雌二醇能使乙酰胆碱及氯化钙的量效曲线明显右移（pD_2' 值分别为 3.98 和 4.75）. 另外，雌二醇可明显抑制乙酰胆碱引起的第 I 时相性收缩，对氯化钙引起的第 II 时相性的收缩无明显影响. (2) 肌注雌二醇 (1 mg/kg) 可使豚鼠的引喘潜伏期明显延长. **结论**：(1) 雌二醇对兔离体气管平滑肌的作用是非上皮依赖性的，与抑制电压依赖性钙通道和细胞内钙从内质网的释放有关，还部分与 cGMP 介导的松弛途径及刺激气道平滑肌释放前列腺素类物质有关，但与 β-肾上腺素能受体介导的舒张无关. (2) 雌二醇可明显舒张豚鼠在体气管平滑肌.

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